conditions sufficient to permit detectable binding of said probe to said target, and

b) detecting the presence or absence, and optionally the amount, of said binding.

REMARKS

Status of the claims

Claims 1 - 56, as originally filed, are pending.

Claims 16 - 32, 37 - 44, 46 - 48, and 52 - 56 have been withdrawn from consideration, and claims 1 - 15, 33 - 36[1], 45, and 49 - 51 stand rejected.

Applicants herein cancel without prejudice claims 1 - 56 and add new claims 57 - 75. New claims 57 - 75 fall within the restriction group earlier elected for prosecution on the merits. Support for the claims newly added by amendment may be found throughout the specification, including the claims as originally filed. No new matter has been added.

Response to formal matters

Specification

References to hyperlinks in the specification have been amended to provide instead a description of the organization hosting the relevant web sites.

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[1] Applicants note that references to claims "33-369" at several places in the office action are clearly in error and are presumed instead to refer to claims "33-36".

The blanks left in the specification for the biological deposit accession number have been completed by amendment. Support for these amendments may be found in the letter from the ATCC, a copy of which is filed herewith, noting the patent deposit designation for the deposited strain.

Applicants respectfully submit that no new matter has been added by these amendments to the specification.

Claim objections

The objection to claim 49 has been rendered moot by the cancellation of this claim. New claims 57 - 75 are all drawn to elected subject matter.

Rejections Under 35 U.S.C. § 101 For Lack Of Utility Have Been Obviated By Amendment And/Or Are In Error And Should Be Withdrawn

Claims 1 - 15, 33 - 36, 45, and 49 - 51 stand rejected under 35 U.S.C. § 101 as not being supported by either a specific or substantial asserted utility or a well established utility.

Applicants respectfully traverse the rejection.

Solely to expedite prosecution, however, and without admitting to the adequacy of the Examiner's *prima facie* case of unpatentability, applicants have cancelled all of the rejected claims, formally obviating the rejection. Applicants respectfully submit, however, that the rejection would be in error if reasserted against the claims newly added by amendment herein and request that the rejection be withdrawn.

Applicants first note that the utility requirement of § 101 is met either if the claimed subject matter has a "well-established" utility, or if a substantial, specific, and credible utility is disclosed in the specification.

> An invention has a well-established utility (1) if a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (*e.g.*, properties or applications of a product or process), and (2) the utility is specific, substantial, and credible.

Utility Examination Guidelines, 66 Fed. Reg. 1092, 1098 (Jan. 5, 2001). For example, "some uses can be immediately inferred from a recital of certain properties." *In re Folkers*, 344 F.2d 970, 974 (C.C.P.A. 1965) (explicitly undisturbed by *Brenner* v. *Manson*, 383 U.S. 519, 535 n.23 (1966) and *In re Kirk*, 376 F.2d 936, 949 (C.C.P.A. 1967) (Rich, J., dissenting)). In particular, when "newly discovered compounds [that] belong to a class of compounds, the members of which have become well recognized as useful for a particular purpose because of a particular property, the only reasonable conclusion is that the new compounds, also possessing that property, are similarly useful." *Folkers* at 975, *see also* MPEP 2107.02.

In the instant application, claimed subject matter comprises nucleotide sequences encoding a novel protein that contains a predicted HR1 motif. *See* p. 132 and Figs. 1A - B of the application. Other proteins containing this motif were well known at the time the application was filed to bind specifically to a small GTPase, Rho. *See* p. 3 of the specification; Reid T. et al., *J. Biol. Chem.* 271:13558-13560 (1996) (copy enclosed). Also well known at the time the

application was filed was the use of a nucleotide sequence encoding a Rho binding protein to generate a fusion protein containing the Rho binding domain and the use of the fusion protein as an affinity agent for binding GTP-bound Rho. *Id.* Such a fusion protein could also be used as an inhibitor of both endogenous and GTPase-activating protein-stimulated Rho GTPase activity. *Id.*

The instantly claimed nucleotide sequences thus belong to a class of compounds that display utility by their use in the generation of affinity reagents and enzyme inhibitors. The utility of this class of nucleotide sequences is further evidenced by the commercial availability of fusion proteins generated from these sequences. As the enclosed printout from Pierce Biotechnology shows, GST-fusion proteins containing the binding domains of various GTPase binding proteins--including Rhotekin, a mouse Rho binding protein--are currently sold in kits costing over $500. The other printouts show that affinity beads containing the Rhotekin fusion protein are commercially available for $250 per 500 μg. In a utility analysis, the real world immediate benefit to the public may be monetary rather than medicinal, pecuniary rather than principled. Indeed, "a patent system **must** be related to the world of commerce," *Brenner* v. *Manson*, 383 U.S. 519, 536 (1966) (*quoting In re Ruschig*, 343 F.2d 965, 970 (C.C.P.A. 1965)) (emphasis added), and commercial utilities are thus paradigmatic "real-world" contexts that establish substantial utility. Indeed, a finding that an otherwise valid patent claim is infringed, such as by the sale of the claimed invention by a party other than the patent owner, is

dispositive proof, as a matter of law, of the utility of the claim. *Raytheon* v. *Roper*, 724 F.2d 951, 959 (Fed. Cir. 1983); *United States Steel Corp.* v. *Phillips Petroleum Co.*, 865 F.2d 1247, 1252 (Fed. Cir. 1989).

Because the claimed nucleotide sequences of the instant application belong to a class of compounds, the members of which have well-established utility, Applicants respectfully submit that the claimed nucleotide sequences, which are also capable of these particular purposes, are similarly useful. According to the Federal Circuit, "[t]he threshold of utility is not high: An invention is 'useful' under section 101 if it is capable of providing **some identifiable benefit**." *Juicy Whip, Inc.* v. *Orange Bang, Inc.*, 185 F.3d 1364, 1366 (Fed. Cir. 1999) (emphasis added).

Rejections Under 35 U.S.C. § 112, ¶ 1 For Lack Of Enablement Have Been Obviated By Amendment And/Or Are In Error And Should Be Withdrawn

Claims 1 - 15, 33 - 36, 45, and 49 - 51 stand rejected under 35 U.S.C. § 112, ¶ 1. According to the Examiner, since the claimed invention is not supported by a specific or substantial utility or a well-established utility, the disclosure also fails to enable one skilled in the art to make and use the invention. Claims 33 - 36 and 49 are additionally rejected under 35 U.S.C. § 112, ¶ 1 as not enabled for their full scope, either for pharmaceutical or *in vivo* use.

Applicants respectfully traverse the rejection.

Solely to expedite prosecution, however, and without admitting to the adequacy of the Examiner's *prima facie* case of

unpatentability, applicants have cancelled all of the rejected claims, formally obviating the rejection. Applicants respectfully submit, however, that because the claims newly added herein indeed display a well-established utility for the reasons advanced above, the derivative rejection for non-enablement would be in error if reasserted against these claims. Applicants respectfully request therefore that the rejection be withdrawn.

Written Description Rejections Under 35 U.S.C. § 112, ¶ 1 Have Been Obviated By Amendment And/Or Are In Error And Should Be Withdrawn

Claims 1 - 15, 33 - 36, 45, and 49 - 51 stand rejected under 35 U.S.C. § 112, ¶ 1, as containing subject matter that is not adequately described in the specification.

Applicants respectfully traverse the rejection.

Solely for the sake of expediency, however, and without admitting to the adequacy of the Examiner's *prima facie* case of unpatentability, Applicants have cancelled all of the rejected claims, formally obviating the rejection, and in the claims newly added by amendment herein have avoided the phrase "conservative amino acid substitutions" that the Examiner suggests was not sufficiently described in the specification.

Applicants respectfully submit that the genera now claimed are fully supported by the specification and that the rejection should be withdrawn.

Rejections Under 35 U.S.C. § 112, ¶ 2 For Indefiniteness Have Been Obviated By Amendment And Should Be Withdrawn

Claims 1 - 15, 33 - 36, 45, and 49 - 51 stand rejected under 35 U.S.C. § 112, ¶ 2, as being indefinite for failing to particularly point out and distinctly claim the subject matter regarded as the invention.

Applicants have cancelled all of the rejected claims, formally obviating the rejection, and in the claims newly added by amendment herein have avoided the indefinite format noted by the Examiner in this rejection. The rejections having been obviated, Applicants respectfully request that the rejection be withdrawn.

Rejections under 35 U.S.C. § 102 For Anticipation Have Been Obviated By Amendment And Should Be Withdrawn

Claim 1 has been rejected as anticipated by Burbelo et al., Wu et al., and DOE Joint Genome Institute with respect to limitations vi (at least 17 contiguous nucleotides of SEQ ID NO:4), vii (at least 17 contiguous nucleotides of SEQ ID NO:6), and viii (a nucleotide sequence that encodes a polypeptide having the sequence of SEQ ID NO:7) and by Babij et al. with respect to limitation vii (at least 17 contiguous nucleotides of SEQ ID NO:6). Claims 1, 4 -6, 8 - 11, and 33 - 36 have been rejected as anticipated by Sonstegard et al. with respect to limitation vi of claim 1, and the further limitations of claims 4 - 6, 8 - 11, and 33 - 36.

Solely to expedite prosecution, Applicants have cancelled all of the rejected claims, formally obviating the

rejection, and in the claims newly added by amendment herein have omitted the above-recited limitations. The rejections having been obviated, Applicants respectfully request that the rejection be withdrawn.

Rejections under 35 U.S.C. § 103 For Obviousness Have Been Obviated By Amendment And Should Be Withdrawn

Claims 4 - 6, 8 - 15, 33 - 36 stand rejected under 35 U.S.C. 103(a) as unpatentable over Babij et al. in view of Sambrook. According to the Examiner, Babij et al. teaches the cDNA sequence of a rabbit myosin heavy chain. This sequence contains an 18 nucleotide segment that is also found within SEQ ID NO:6 of the instant application and that therefore anticipates the limitation in claim 1 to a nucleotide sequence with at least 17 contiguous nucleotides of SEQ ID NO:6. The Examiner asserts that although Babij does not specifically teach placing the nucleic acid in a vector and host cell, Sambrook teaches methods for expressing large amounts of protein from cloned genes introduced into E. coli, and that expression has proven invaluable in the purification, localization and functional analysis of proteins. According to the Examiner, it would have been *prima facie* obvious to one of ordinary skill in the art to insert the nucleic acid of Babij et al. into a vector and host cell to produce large amounts of protein as taught by Sambrook.

Applicants have cancelled all of the rejected claims, formally obviating the above rejections, and in the claims newly added by amendment herein have omitted the limitation

from claim 1 as filed to a nucleotide sequence with at least 17 contiguous nucleotides of SEQ ID NO:6. Applicants submit that absent this limitation, the newly filed claims would not be *prima facie* obvious over Babij et al. in light of Sambrook. The rejections having been obviated, Applicants respectfully request that the rejection be withdrawn.

Claims 7, 45, and 49 - 50 also stand rejected under 35 U.S.C. § 103(a) as obvious over Burbelo et al., Wu et al., DOE Joint Genome Institute, Sonstegard et al., or Babij et al. in view of DeRisi et al. According to the Examiner, Burbelo et al. teaches a human mRNA sequence comprising nucleotides 1-89 of SEQ ID NO:4, and Wu et al. and DOE Joint Genome Institute both teach a human chromosomal clone comprising this same sequence. According to the Examiner, these references therefore include at least 17 contiguous nucleotides of SEQ ID NO:4 (limitation vi of claim 1), at least 17 contiguous nucleotides of SEQ ID NO:6 (limitation vii of claim 1), and a nucleic acid encoding SEQ ID NO:7 (limitation viii of claim 1). According to the Examiner, Sonstegard et al. teaches a bovine sequence including nucleotides 37-64 of SEQ ID NO:4 (limitation vi of claim 1) as well as the further limitations of claims 4 - 6 and 8 - 11. Finally, as noted above, the Examiner notes that Babij et al. teaches a rabbit myosin heavy chain mRNA sequence corresponding to nucleotides 35-52 of SEQ ID NO:6 (limitation vii of claim 1).

Although none of the above references teaches attaching the nucleic acid to a solid support or labeling the nucleic acid, the Examiner notes that DeRisi et al. teaches the use of cDNA microarrays to analyze gene expression patterns and

also teaches labeling nucleic acids prior to their analysis on a solid support. The Examiner suggests that it would have been *prima facie* obvious to one of ordinary skill in the art to modify the nucleic acids taught by the above references and to place these nucleic acids on a solid support according to the teachings of DeRisi et al.

Applicants have cancelled all of the rejected claims, formally obviating the above rejections, and in the claims newly added by amendment herein have omitted limitations from claim 1 as filed to a nucleotide sequence with at least 17 contiguous nucleotides of SEQ ID NO:4, to a nucleotide sequence with at least 17 contiguous nucleotides of SEQ ID NO:6, and to a nucleotide sequence that encodes a polypeptide having the sequence of SEQ ID NO:7. Applicants submit that absent these limitations, the newly filed claims would not be *prima facie* obvious over Burbelo et al., Wu et al., DOE Joint Genome Institute, Sonstegard et al., or Babij et al. in light of DeRisi. The rejections having been obviated, Applicants respectfully request that the rejection be withdrawn.

CONCLUSION

Applicants respectfully submit that claims 57 - 75, all of the claims now pending, are free of rejection. Applicants thus respectfully submit that the claims are in good and proper form for allowance, and respectfully request the same.

If the Examiner believes that any issues remain outstanding, Applicants respectfully request that the Examiner call the undersigned for a telephonic discussion.

Respectfully submitted,

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I hereby Certify that this Correspondence is being Deposited with the U.S. Postal Service First Class Mail in an Envelope Addressed to: Hon. Commissioner for patents P.O. Box 2327. Arlington, VA 22202, on

02-05-2003

LILY X. JIANG

Signature of Person Signing

Enclosures:

1) A copy of the letter from ATCC documenting original deposit of a plasmid containing 4 cDNA fragments of Human GTP-RHO binding protein 2 pursuant to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure;

2) A printout of the web page describing the "EZ-Detect Rho Activation Kit" from Pierce Biotechnology;

3) A printout of the web page describing "Rhotekin-RBD Protein GST Beads" from Cytoskeleton, Inc.;

4) A printout of the web page describing "Rhotekin Rho Binding Domain, agarose" from Upstate Cell Signaling Solutions;

5) A copy of Reid et al., "Rhotekin, a New Putative Target for Rho Bearing Homology to a Serine/Threonine Kinase, PKN, and Rhophilin in the Rho-binding Domain", J. Biol. Chem. 271:13556-13560 (1996);

6) An associate power of attorney appointment.

Amendments to the Specification

Marked-Up Pursuant to 37 C.F.R. § 1.121(b)(1)(iii)

(1) Replacement for the paragraph beginning at line 28 on p. 18 and continuing to line 8 on p. 19:

Accordingly, four overlapping cDNA clones that together can be used to provide an assembled consensus sequence spanning the GRBP-2 cDNA were deposited in a public repository (American Type Culture Collection, Manassas, Virginia, USA) on June 27, 2001 and collectively been assigned accession no. PTA-3484[_____]. Clone 1 (designation grbp2-5r1) contains nucleotides 1 - 742 (numbering as in FIG. 3), clone 2 (designation grbp2-rt1) contains nucleotides 419 - 1360, clone 3 (grbp2-3f13) contains nucleotides 724 - 2748, and clone 4 (grbp2-rt5) contains nucleotides 1314 - 3489, plus the poly-A tail. Any errors in sequence reported herein can be determined and corrected by sequencing nucleic acids propagated from the deposited clones using standard techniques.

(2) Replacement for the paragraph on p. 20 starting at line 3 and ending at line 10:

For purposes herein, percent identity of two nucleic acid sequences is determined using the procedure of Tatiana *et al.*, "Blast 2 sequences - a new tool for comparing protein and nucleotide sequences", FEMS Microbiol Lett. 174:247-250 (1999), which procedure is effectuated by the computer program BLAST 2

SEQUENCES, available online at the National Center for Biotechnology Information (NCBI) website.[http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2 .html.]

(3) Replacement for the paragraph on p. 53 starting at line 10 and ending at line 20:

In a first such embodiment, the invention provides an isolated nucleic acid comprising (i) the assembled consensus nucleotide sequence of the four overlapping cDNAs deposited at the ATCC on June 27, 2001 and collectively accorded accession no. PTA-3484[_____], (ii) the nucleotide sequence of SEQ ID NO: 1, or (iii) the complement of (i) or (ii). The assembled consensus nucleotide sequence of the four overlapping nucleic acids of the ATCC deposit has, and SEQ ID NO: 1 presents, the entire cDNA of human GRBP2, including the 5' untranslated (UT) region and 3' UT.

(4) Replacement for the paragraph on p. 78 starting at line 6 and ending at line 14:

Bacterial cells can be rendered electrocompetent – that is, competent to take up exogenous DNA by electroporation – by various pre-pulse treatments; vectors are introduced by electroporation followed by subsequent outgrowth in selected media. An extensive series of protocols is provided online in Electroprotocols Online: Collection of Protocols for Gene Transfer (Bulletin #1029735, BioRad,

Richmond, CA, USA)[(http://www.bio-rad.com/LifeScience/pdf/New_Gene_Pulser.pdf)].

(5) Replacement for the paragraph on p. 81 starting at line 23 and ending at line 32:

For purposes herein, percent identity of two amino acid sequences is determined using the procedure of Tatiana *et al.*, "Blast 2 sequences - a new tool for comparing protein and nucleotide sequences", FEMS Microbiol Lett. 174:247-250 (1999), which procedure is effectuated by the computer program BLAST 2 SEQUENCES, available online at the National Center for Biotechnology Information (NCBI) website.[

http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html,

]

(6) Replacement for the paragraph starting at line 27 on p. 102 and ending at line 6 on p. 103:

In a first series of protein embodiments, the invention provides an isolated human GRBP2 polypeptide having an amino acid sequence encoded by the assembled consensus nucleotide sequence of the four overlapping cDNAs deposited at the ATCC on June 27, 2001 and collectively accorded accession no. PTA-3484[_____], or the amino acid sequence in SEQ ID NO: 3, which are full length human GRBP2 proteins. When used as immunogens, the full length proteins of the present invention can be used, inter alia, to elicit antibodies that

bind to a variety of epitopes of the several forms of human GRBP2 protein.

(7) Replacement for the paragraph starting on p. 119 at line 10 and ending at line 18:

In a first series of antibody embodiments, the invention provides antibodies, both polyclonal and monoclonal, and fragments and derivatives thereof, that bind specifically to a polypeptide having an amino acid sequence encoded by the assembled consensus of the four cDNAs deposited in the ATCC on June 27, 2001 and collectively accorded accession no. PTA-3484[_____], or that have the amino acid sequence in SEQ ID NO:3, which are full length human GRBP2 proteins.

(8) Replacement for the paragraph starting on p. 129 at line 13 and ending at line 16:

The human GRBP2 cDNA was deposited at the American Type Culture Collection (ATCC) on June 27, 2001 as four overlapping cDNA fragments collectively accorded accession number PTA-3484[_____].

(9) Replacement for the paragraph starting at line 30 on p. 131 and continuing to line 2 on p. 132:

Motif searches using Pfam (Washington University, St. Louis, web site[http://pfam.wustl.edu]), SMART (European Molecular Biology Laboratory, Heidelberg, web

site [http://smart.embl-heidelberg.de]), and PROSITE pattern and profile databases (Expert Protein Analysis System (ExPASy) web site [http://www.expasy.ch/prosite]), identified several known domains shared with mouse Grbp1 and Grbp2.

(10) Replacement for the paragraph starting at line 32 on p. 132 and continuing to line 4 on p. 133:

Transcription factor binding sites were identified using MOTIF, available at the GenomeNet web site (Bioinformatics Center, Institute for Chemical Research, Kyoto University) [a web based program (http://motif.genome.ad.jp/)], including a binding site for MZF1 (917-924 and 927-934 bp), for cap (cap signal for transcription initiation, 969-976 and 983-990 bp), for SP1 (836-845, 915-924, and 937-946 bp, with numbering according to SEQ ID NO: 38), amongst others.

ATCC

10801 University Blvd • Manassas, VA 20110-2209 • Telephone: 703-365-2700 • FAX: 703-365-2745

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Aeomica, Inc.
Attn: David Hanzel
928 East Arques Ave.
Sunnyvale, CA 94085

Deposited on Behalf of: Aeomica, Inc.

Identification Reference by Depositor:	**Patent Deposit Designation**
Plasmid in E. coli: Human GTP-RHO binding protein 2:4 cDNAs fragments	PTA-3484

The deposit was accompanied by: __ a scientific description __ a proposed taxonomic description indicated above.

The deposit was received June 28, 2001 by this International Depository Authority and has been accepted.

AT YOUR REQUEST: X We will inform you of requests for the strain for 30 years.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strain.

If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with living culture of the same.

The strain will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested July 13, 2001. On that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Manassas, VA 20110-2209 USA.

Signature of person having authority to represent ATCC:

Date: July 30, 2001

Tanya Nunnally, Patent Specialist, Patent Depository

cc: Daniel Becker
(Ref: Docket or Case No.: AEOMICA-11)

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Rhotekin, a New Putative Target for Rho Bearing Homology to a Serine/Threonine Kinase, PKN, and Rhophilin in the Rho-binding Domain*

(Received for publication, January 3, 1996, and in revised form, March 1, 1996)

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Using a mouse embryo cDNA library, we conducted a two-hybrid screening to identify new partners for the small GTPase Rho. One clone obtained by this procedure contained a novel cDNA of 291 base pairs and interacted strongly with RhoA and RhoC, weakly with RhoB, and not at all with Rac1 and Cdc42Hs. Full-length cDNAs were then isolated from a mouse brain library. While multiple splicing variants were common, we identified three cDNAs with an identical open reading frame encoding a 61-kDa protein that we named rhotekin (from the Japanese "teki," meaning target). The N-terminal part of rhotekin, encoded by the initial cDNA and produced in bacteria as a glutathione *S*-transferase fusion protein, exhibited *in vitro* binding to ^{35}S-labeled guanosine 5′-3-*O*-(thio)triphosphate-bound Rho, but not to Rac1 or Cdc42Hs in ligand overlay assays. In addition, this peptide inhibited both endogenous and GTPase-activating protein-stimulated Rho GTPase activity. The amino acid sequence of this region shares ~30% identity with the Rho-binding domains of rhophilin and a serine/threonine kinase, PKN, two other Rho target proteins that we recently identified (Watanabe, G., Saito, Y., Madaule, P., Ishizaki, T., Fujisawa, K., Morii, N., Mukai, H., Ono, Y., Kakizuka, A., and Narumiya, S. (1996) *Science* 271, 645–648). Thus, not only is rhotekin a novel partner for Rho, but it also belongs to a wide family of proteins that bear a consensus Rho-binding sequence at the N terminus. To our knowledge, this is the first conserved sequence for Rho effectors, and we have termed this region Rho effector motif class 1.

The Ras superfamily of small GTPases encompasses a group of ubiquitous regulatory proteins related by both structure and function. The products of such genes are involved in a plethora of intracellular signaling processes (1). These proteins are generally regarded as being activated in the GTP-bound form. The intrinsic hydrolytic activity of these proteins is responsible for the reversion to the resting GDP-bound form. Proteins of the Rho subfamily play a pivotal role in the regulation of cytoskeletal organization and the determination of cell polarity. Strongly linked to the formation of stress fibers and focal adhesions (2), regulation of cell motility (3), aggregation (4, 5), cell cycle progression (6), and contractile ring formation and cytokinesis (7, 8), the Rho proteins occupy key positions in many fundamental cellular processes.

A large number of regulatory proteins for Rho have been characterized, including nucleotide exchange proteins (9–11), GTPase-activating proteins (GAPs)[1] (12, 13), and guanine nucleotide dissociation inhibitors (14, 15). In contrast, there has been surprisingly little information available on the nature of the molecules that are directly regulated by Rho. Recently, Rho has been proposed to regulate phosphatidylinositol-4-phosphate 5-kinase and to regulate actin polymerization through increases in phosphatidylinositol 4,5-bisphosphate levels (16). However, a direct interaction with a regulatory element that may give rise to this effect has yet to be demonstrated.

The two-hybrid system was used successfully to demonstrate *in vivo* interactions between Ras and its downstream effectors, Byr-1 (17), Raf-1, and CYR1 (18). More recently, this system has been used to dissect precisely which features of Ras are involved in interactions with multiple effectors and how this contributes to oncogenesis (19). To isolate and examine downstream Rho targets, we conducted a library screening using the yeast two-hybrid system and complemented our data with *in vitro* confirmation of the interaction. By these procedures, we have identified a Ser/Thr protein kinase (PKN), a PKN-related protein (rhophilin), and a 180-kDa coiled coil-containing protein (citron) as potential Rho target molecules (20, 21). We have also isolated a novel Ser/Thr protein kinase, p160^{ROCK}, as a potential Rho effector (22) that displays a structural similarity to citron. The present report describes the isolation of a new putative target protein that binds to the GTP-bound form of Rho and inhibits its GTPase activity. The Rho-binding region of this protein appears to be related to those of PKN and rhophilin.

EXPERIMENTAL PROCEDURES

Materials—[^{35}S]GTPγS (1000 Ci/mmol), [^{35}S]GDPβS (1000 Ci/mmol), and [γ-^{32}P]GTP (6000 Ci/mmol) were obtained from DuPont NEN. Plasmids pGEX-*rhoA* (23), pGEX-*rac1* (24), and pGEX-*CDC42Hs* (25) (gifts of Dr. Yoshimi Takai, Osaka University, Osaka, Japan) and pGEX-KG-*rhoGAP* (26) (a gift of Dr. Alan Hall, University College,

* This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan and by grants from the Human Frontier Science Program, the Senri Life Science Foundation, and the Naito Memorial Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U54638.

‡ Supported by a postdoctoral fellowship from the Japan Society for the Promotion of Science. Present address: Faculté de Pharmacie, Université Paris-Sud, INSERM CJF 93-01, 5 Rue Jean Baptiste Clément, 92296 Chatenay Malabry Cedex, France.

§ On leave from CNRS (France) and supported by the Japan Society for the Promotion of Science.

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[1] The abbreviations used are: GAPs, GTPase-activating proteins; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; GDPβS, guanosine 5′-(β-thio)diphosphate; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; VAD, VP16 transcription activation domain; REM-1, Rho effector motif class 1.

London) were expressed in *Escherichia coli* as GST fusion proteins and were prepared as described (23). Plasmids pVP16 and pBTM116 for use in the two-hybrid system were gifts of Drs. Stan Hollenberg, Rolf Sternglanz, Stan Fields, and Paul Bartel.

Yeast Two-hybrid System Screening—Two-hybrid system screening was conducted essentially as described previously (18), except that strain AMR70 was used in conjunction with L40 in the mating strategy. The initial screening was conducted with a RhoC mutant with a deletion at residue 181, lacking the C*AAX* box and the polybasic region. Deletion was carried out through PCR amplification using the original *rhoC* cDNA (27) as a template. This PCR, using a 5′-end primer of AGCGGATCCATGGCTGCAATCCGAAAGAAG and a 3′-end primer of CCAGAATTCAGACCTGGAGGCCAGCCCGAG, introduced a *Bam*HI site at the 5′-end before codon 1 and a *Eco*RI site at the 3′-end after codon 181. This cDNA was then subcloned into the multiple cloning site of a modified pBTM116 plasmid (pBTM116M) (21). This vector was called pBTM116M-*rhoCΔC* and drove the expression of a LexA-RhoCΔC fusion protein. Similar deletions were made also by PCR for *rhoA* using a 5′-end primer of AGCGGATCCATGGCTGCCATCCGGAAGAAA and a 3′-end primer of CCAGAATTCAAGCTTGCAGAGCTCTCG and for *rhoB* with a 5′-end primer of AGCGGATCCATGGCGGCCATCCGCAAGAAG and a 3′-end primer of CCAGAATTCACTTCTGCAGCGCGGCGCGCG with *rhoA* cDNA (23) and *rhoB* cDNA (27) as templates, respectively; the resulting cDNAs were inserted similarly to pBTM116M. Full-length *rac1* and *CDC42Hs* cDNAs were excised from the respective pGEX plasmid DNA with *Bam*HI and inserted into pBTM116M to produce LexA-Rac1 and LexA-Cdc42, respectively. A murine day 10.5 embryonic library in pVP16 (18) was screened with a bait plasmid featuring LexA fused to a RhoC deletion mutant (pBTM116-*rhoCΔC*). These clones were then used directly for the analysis of LacZ expression. From 1.2×10^7 initial transformants, we identified 256 $LacZ^+$ histidine prototrophs, 79 of which were cured of pBTM116-*rhoCΔC*. Interactions with other proteins were evaluated after mating with yeast strain AMR70 harboring various test baits. Of the 79 cured clones, 22 were $LacZ^+$ with the initial screening bait and $LacZ^-$ with the lamin fusion construct. Of these, 12 clones appeared to carry pVP16 containing the same cDNA insert. A 291-base pair insert was excised from the plasmid of clone 21 and designated as C21.

cDNA Cloning of the Full-length Rhotekin—A murine brain oligo(dT)-primed cDNA library in λZAP II (Stratagene) was used to isolate a full-length rhotekin cDNA. A total of 1.1×10^6 independent clones were screened on nylon filter membranes (DuPont NEN PlaqueScreen) by hybridization with a ^{32}P-labeled C21 cDNA. Hybridization of the probe and subsequent washing of filters were carried out as described (28). Positives were rescreened once, and plasmid DNA was rescued using XL-1 Blue *E. coli* and helper phage VCS M13 (Stratagene) according to the manufacturer's instructions. Nucleotide sequencing was carried out on both strands by the use of the dideoxy chain termination method. To examine the interaction of the full-length rhotekin in the two-hybrid system, the full coding sequence of rhotekin cDNA from the *Fsp*I site to the 3′-*Xho*I site in the multiple cloning site of pBluescript SK was inserted in the *Not*I site of pVP16 to create plasmid pVP16-rhotekin (full length).

Northern Blotting—Total RNA was isolated from dissected murine tissues as described (28), and poly(A)$^+$ RNA was purified using oligo(dT)-latex beads (Pharmacia Biotech Inc.). Two μg poly(A)$^+$ RNA was separated by electrophoresis on a 1.2% formaldehyde-agarose gel, transferred to a nylon membrane, and immobilized by UV cross-linking. The RNA was then hybridized with a ^{32}P-labeled *Xho*I-*Xho*I fragment of the full-length rhotekin cDNA in 50% formamide, 5 × SSC, 50 mM Tris·HCl, pH 7.5, 5 × Denhardt's solution, 0.1% SDS, and 200 μg/ml yeast RNA at 42 °C for 16 h. The filter was washed finally with 0.5 × SSC and 0.1% SDS at 65 °C and analyzed using a Fuji BAS2000 Bioimage analyzer.

Ligand Overlay Assays—Ligand overlay assays were employed as an *in vitro* confirmation of positives. The insert from pVP16-C21 was transferred to pGEX-3X to give pGEX-C21. pGEX-rhotekin (amino acids 7–113) was created by introducing the rhotekin coding sequence between *Fsp*I and *Bam*HI sites into pGEX-3X. The plasmids were expressed as GST fusion proteins in bacteria, and 5 μg of protein of total bacterial lysate was subjected to SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell). The adherent proteins were renatured on the filter and then incubated with radiolabeled small GTPase as described (21, 29). Each of the small GTPases was preloaded either with [^{35}S]GTPγS or with [^{35}S]GDPβS (both at 1000 Ci/mmol). The bound radioactivity was determined by filter assay, and a 5 nM concentration of the radiolabeled protein was added to the incubation. After incubation, the filter was washed briefly and rapidly dried. Interactions were imaged by autoradiography.

GAP Protection Assay—GAP protection was carried out essentially as described previously (29, 30). GST-RhoA (80–100 pmol) was first loaded with [γ-^{32}P]GTP (30 Ci/mmol) in buffer A (20 mM Hepes/NaOH, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 1 mg/ml bovine serum albumin, and 10 mM dithiothreitol). The intrinsic rate of GTP hydrolysis was examined by incubating 20 pmol of [^{32}P]GTP-RhoA in 50 μl of buffer B (20 mM Hepes/NaOH, pH 7.5, 1 mM $MgCl_2$, and 1 mg/ml bovine serum albumin) at 30 °C. Duplicate aliquots of 5 μl were removed after 0, 2.5, 5, 10, and 15 min and applied to a BA85 membrane. The amount of RhoA-bound [^{32}P]GTP remaining unhydrolyzed after each incubation was determined by the amount of radioactivity adhering to the filter. The GAP-catalyzed rate of GTP hydrolysis was examined in the presence of 1 μg of purified GST-rhoGAP. The effect of GST-C21 on the rate of intrinsic and GAP-stimulated GTP hydrolysis was determined by preincubating [^{32}P]GTP-RhoA with 5 μg of GST-C21 on ice for 5 min prior to transfer to a bath at 30 °C. The dose dependence of the inhibitory effect of GST-C21 on the intrinsic and GAP-catalyzed GTPase activity of RhoA was determined by preincubating 0, 1, 2.5, 5, 7.5, or 10 μg of GST-C21 with 8 pmol of GTP-RhoA in 50 μl of buffer B for 5 min on ice, followed by transfer to a bath at 30 °C for 5 min, with or without the addition of 0.5 μg of GST-rhoGAP.

RESULTS AND DISCUSSION

We conducted a library screening with the yeast two-hybrid system in order to identify new partners for Rho. The bait vector pBTM116 drives the expression of the LexA transcription factor fused to a bait protein. The complementary plasmid, pVP16, drives the expression of a nuclear localization sequence and the VP16 transcription activation domain (VAD) fused to a random-primed day 10.5 murine embryonic cDNA library. Positive interactions between the bait and proteins expressed from the library plasmid led to the assembly of a transcriptionally active complex driving the expression of yeast *HIS3* and bacterial *lacZ* genes. An initial screening bait was constructed by deleting the C-terminal polybasic region and the C*AAX* box of human RhoC (LexA-RhoCΔC). This strategy was followed on the premise that this region strongly directs Ras and related proteins to the plasma membrane (31, 32) and would interfere with the nuclear localization required for transcriptional activation of the reporter genes in this assay.

We identified several clones displaying the same interaction profile. They appeared to bear the same cDNA in pVP16. Sequencing this insert revealed a novel 291-base pair cDNA, which we called C21. In the two-hybrid system, VAD-C21 was strongly positive with RhoCΔC and RhoAΔC and weaker with RhoBΔC (Fig. 1*A*). Truncation of the C-terminal domains of Rho proteins gave rise to a far stronger interaction than did the full-length forms, possibly either because of a tendency for the C*AAX* box to favor localization to the plasma membrane or through an increased preference of the full-length baits to interact with endogenous yeast proteins. We presume that this reduces the amount of bait protein available for the formation of transcriptionally active complexes. There appeared to be no interaction with either LexA-Rac1 or LexA-Cdc42.

We then expressed the C21 peptide as a bacterial GST fusion protein and examined its interaction with various small GTPases *in vitro* by the ligand overlay assay (Fig. 1*B*). A specific interaction was seen with GTP-RhoA and GTP-RhoB, but not with GDP-RhoA, GTP-Rac1, or GTP-Cdc42. Our attempts to express RhoC as a GST fusion protein were unsuccessful. Taken together, these results are in agreement with the interaction profile observed in the two-hybrid system and clearly demonstrate a specific interaction with GTP-bound Rho proteins. However, due to the method of construction of the library, which included a PCR step, C21 contained one missense PCR mutation and 24 base pairs of the 5′-noncoding region as deduced from the full-length cDNA for rhotekin (see below). To ensure that these did not contribute to or interfere

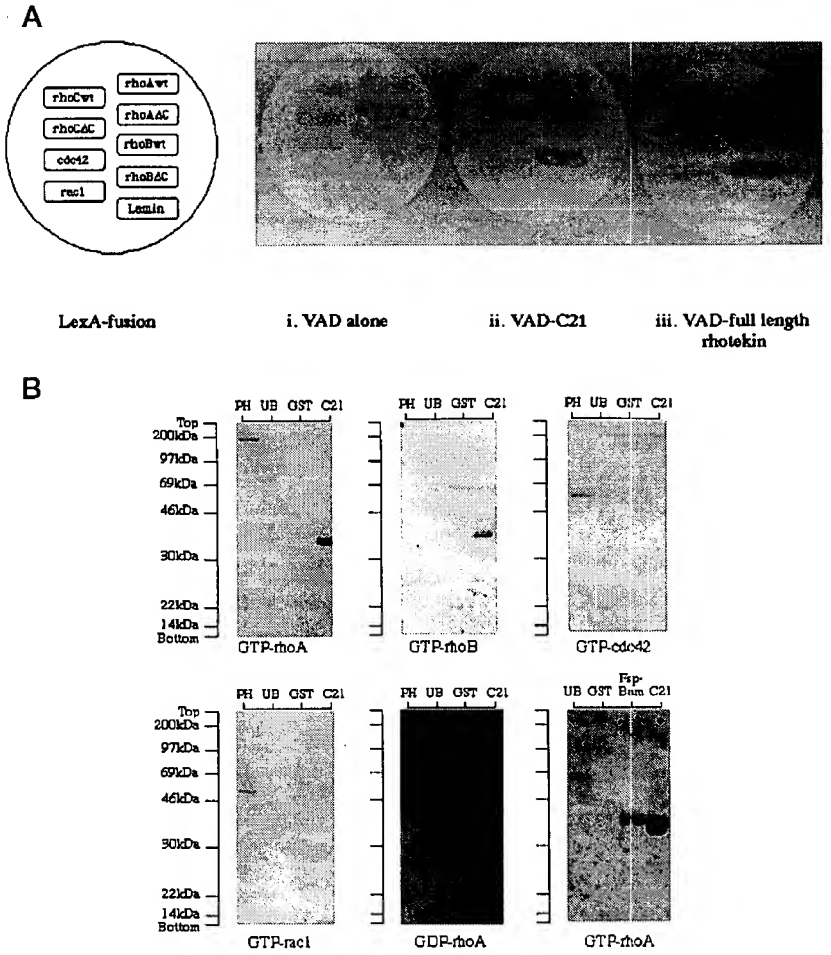

FIG. 1. **Interaction of rhotekin with members of the Rho protein family.** *A, in vivo* two-hybrid system. Shown is the interaction of various LexA fusions with VAD alone (*i*), with VAD-C21 (*ii*), and with VAD-rhotekin (full-length) (*iii*). The same interaction profile was seen with VAD-C21 and VAD-rhotekin (full length). L40 strains harboring the pVP16 vector, pVP16-C21, or pVP16-rhotekin were mated with strain AMR70 expressing various bait constructs in pBTM116. Diploids were cultured as patches on selective medium for both plasmids and transferred to filter papers (Whatman No. 1), and β-galactosidase activity was determined as described (18). *B*, ligand overlay assays. Five μg each of lysates of *E. coli* DH5α without induction (*UB*), expressing GST alone (*GST*), expressing the GST-C21 fusion peptide (*C21*), and expressing GST fused to amino acids 7–113 of full-length rhotekin (*Fsp-Bam*) were subjected to the ligand overlay assay. Renatured proteins were probed with each small GTPase labeled either with [^{35}S]GTPγS (*GTP*) or with [^{35}S]GDPβS (*GDP*). Platelet homogenate (*PH*) was used as a positive control, and a 160-kDa Rho partner and a Rac/Cdc42 partner, presumed to be p160^{ROCK} (22) and p65^{PAK} (29), were detected with [^{35}S]GTPγS-RhoA (*GTP-rhoA*) and [^{35}S]GTPγS-Rac1/Cdc42 (*GTP-cdc42* and *GTP-rac1*), respectively.

with the Rho binding properties of this peptide, we expressed a fragment of the full-length rhotekin containing the N-terminal coding sequence (amino acids 7–113). This peptide was also found to be positive in the overlay assay with GTP-RhoA (Fig. 1*B*). Moreover, when the full-length rhotekin coding region was introduced into pVP16, this, too, displayed an identical interaction profile in the two-hybrid system as did the original cDNA clone (Fig. 1*A*). This indicated that *in vivo* Rho binding activity is a property of the full-length protein as well as the restricted N-terminal fragment.

GST-C21 could be purified from *E. coli* as a soluble protein, allowing us to investigate its effect upon the endogenous and GAP-stimulated GTPase activity of RhoA *in vitro*. We found that this peptide inhibited both endogenous and GAP-stimulated GTP hydrolysis (Fig. 2*A*), and this inhibition occurred in a dose-dependent manner (Fig. 2*B*), indicating that not only does this protein inhibit the interaction of a GAP with Rho, but that it can also modify the inherent hydrolytic activity of the cognate GTPase. Similar interactions between a small GTPase, its effector, and GAP have been reported on Rac1/Cdc42 and p65^{PAK} or Rac1 and p120^{ACK} and their GAP, Bcr (29, 33).

Screening a mouse brain cDNA library using C21 cDNA as a hybridization probe yielded 15 positives from 1.1×10^6 independent clones. Three 2.7-kilobase cDNAs were found to be identical and presumed to be full-length (type 1 cDNA) (Fig. 3*A*). Northern blot analysis of rhotekin mRNA expression using this cDNA as a probe revealed the presence of a transcript of ~3 kilobases in brain and kidney tissues (Fig. 4). Weaker expression was also seen in lung, testis, skeletal muscle, heart,

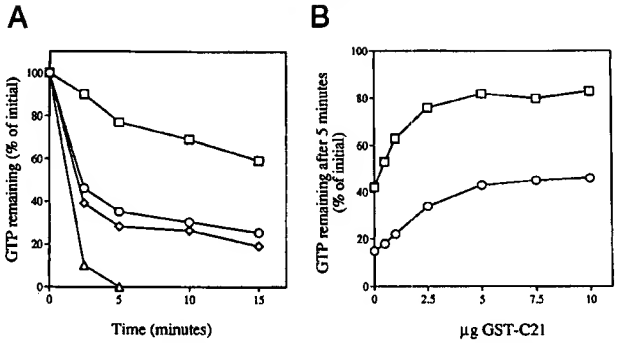

FIG. 2. **GAP protection studies.** *A*, time course. Twenty pmol of [γ-^{32}P]GTP-RhoA was incubated alone (○), with 1 μg of GAP (△), with 5 μg of GST-C21 (□), or with both 1 μg of GAP and 5 μg of GST-C21 (◇), and GTP hydrolysis was determined as described under "Experimental Procedures." *B*, effect of increasing concentrations of GST-C21 on the intrinsic (□) and GAP-stimulated (○) GTPase activity of RhoA. Eight pmol of [γ-^{32}P]GTP-RhoA was incubated for 5 min at 0 °C in the presence of varying amounts of GST-C21. At time 0, the reaction was transferred to 30 °C, and 0.5 μg of GAP was added (○). After 5 min, the remaining [γ-^{32}P]GTP was determined by filter binding assay. The results displayed represent typical results; replicated data varied by ~7%.

and thymus. The size of the transcript appeared to be different in some tissues, and there appeared to be multiple mRNA species in kidney. Consistent with this finding, multiple splicing arrangements were detected also in the brain library, and these inserts appeared also to be full-length (Fig. 3*A*). Type 2

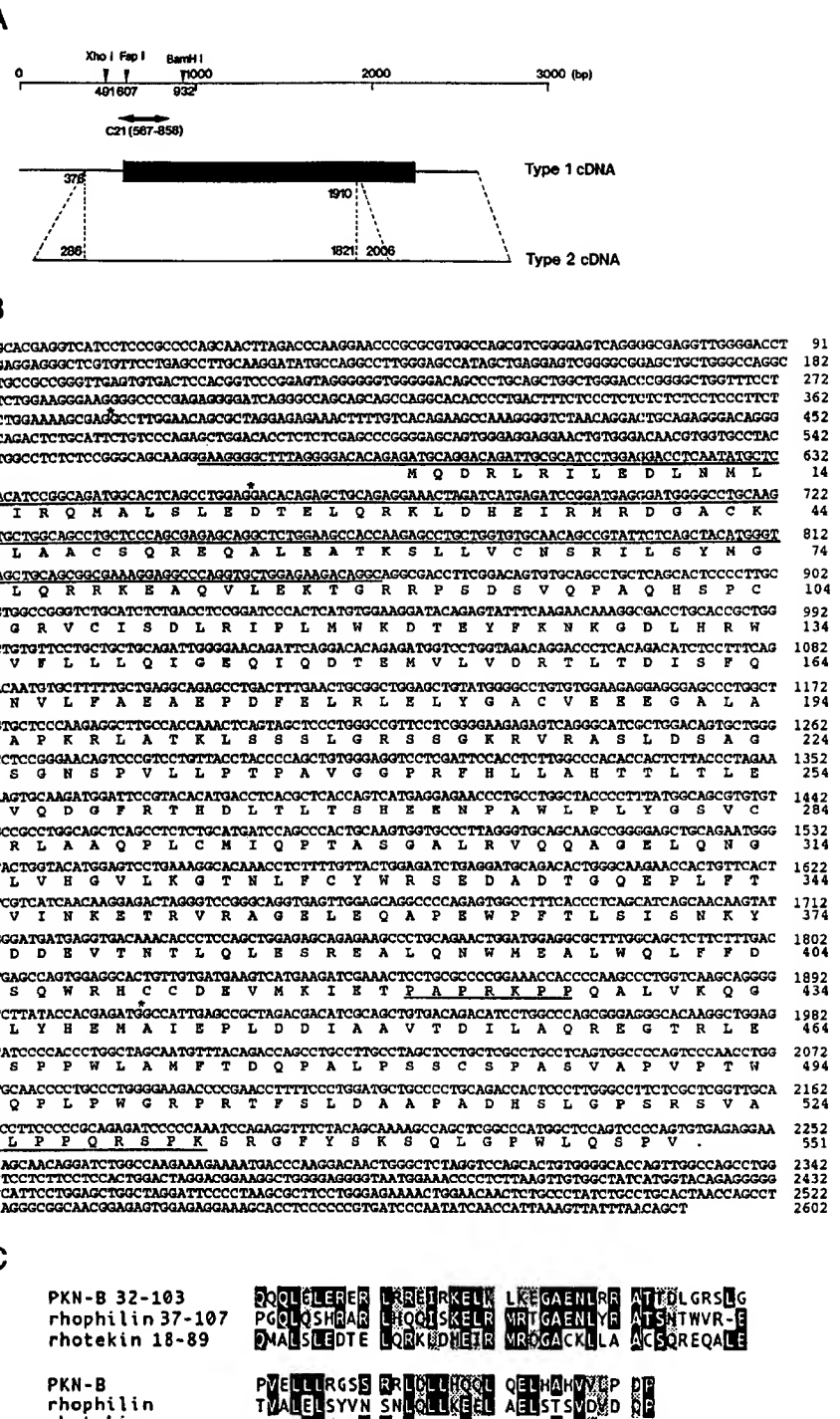

FIG. 3. *A*, schematic representation of the isolated rhotekin cDNAs. The open reading frame is shown by a *closed box*. Restriction enzyme sites and splicing insertion found in type 2 cDNA are shown. *bp*, base pairs. *B*, nucleotide and deduced amino acid sequences of rhotekin. The nucleotide sequence of C21 cDNA and the two C-terminal proline-rich amino acid sequences are *underlined*. Splicing sites are indicated by *asterisks*. *C*, alignment of N-terminal Rho-binding domains of rhotekin, rhophilin, and PKN. Identical residues are indicated in *white* type with a *black* background. Conservative changes in a *shaded* background are grouped as follows: H, K, R; L, I, V; S, T; D, E, N, Q; Y, W; and A, G.

cDNA contains two exon changes at nucleotide 376 (sequence GAG/GC) and at nucleotide 1910 (sequence ATG/GC). The former was localized in the 5′-noncoding region, and the latter caused a 185-base pair insert in the 3′-region of type 1 cDNA. The third splicing variant showed an exon change at nucleotide 662 (sequence GAG/GA) of type 1 cDNA and had a different 5′-end (type 3 cDNA; data not shown). As only one cDNA clone was obtained for each of types 2 and 3, they were not fully characterized. Type 1 cDNA featured a single open reading frame starting at the ATG codon at base 591 and encoding a protein of 551 amino acid residues with a calculated molecular mass of 61 kDa, which we named rhotekin (Fig. 3*B*). Two proline-rich motifs were found toward the C terminus of rhotekin (amino acids 421–427 (PAPRKPP) and amino acids 525–533 (PLPPQRSPK)). Such regions have recently been described as general cognate ligands for numerous SH3 groups (34). C21 cDNA covers nucleotides 567–858, which encodes the rhotekin N-terminal peptide (amino acids 1–89). This, together with the finding obtained with the rhotekin fragment (amino acids 7–113), could locate the Rho-binding domain between amino

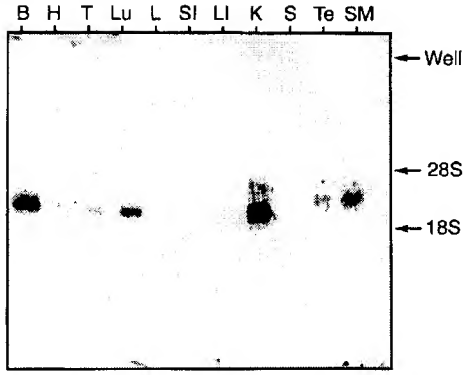

FIG. 4. **Tissue distribution of the rhotekin transcript.** Poly(A)$^+$ RNA was prepared from murine tissues, and 2 μg of each sample was loaded. Full-length rhotekin cDNA was used as template for the probe. Lanes are as follows: *B*, brain; *H*, heart; *T*, thymus; *Lu*, lung; *L*, liver; *SI*, small intestine; *LI*, large intestine; *K*, kidney; *S*, spleen; *Te*, testis; and *SM*, skeletal muscle.

acids 7 and 89. This region showed significant homology to the Rho-binding domains of PKN (35, 36) and rhophilin (Fig. 3*C*) (20). Another common feature is that they are localized in the N terminus of each molecule. However, besides the Rho-binding sites, these proteins are unrelated. Furthermore, data base searches failed to identify any other rhotekin-related proteins. This strongly suggests that this Rho-binding motif is a modular entity that may feature in the regulation of a range of effectors with a spectrum of unrelated activities. We have tentatively termed this domain Rho effector motif class 1 (REM-1). It should be noted that REM-1 has no similarity to the binding motifs of p65^{PAK} and p120^{ACK}, which are the Rac/Cdc42 effectors (29, 33), nor is it present in the coiled coil-bearing Rho effectors such as p160^{ROCK} and citron. Thus, REM-1 may define a particular class of Rho effectors. PKN and rhophilin have been proposed to function as Rho effectors because the kinase activity of PKN is stimulated by Rho binding (20). In addition, as expected for effector molecules for the small GTPases, the REM-1-bearing proteins may inhibit the GTPase activity of Rho, as shown for rhotekin in this study.

Each of the putative Rho target molecules we identified by the two-hybrid system showed some difference in their interaction with Rho proteins in this assay. While the strength of a signal in the two-hybrid system is not an absolute indicator of affinity for interacting molecules, two-hybrid data have been shown to broadly reflect relative affinities for related molecules (37). Rhotekin interacted with RhoC and RhoA equally well (this study), whereas rhophilin interacted exclusively with RhoA (20), and citron acted more preferentially on RhoC (21). These findings may indicate a degree of subtlety and complexity of Rho signaling that different Rho proteins may communicate downstream through different patterns of activation of various effectors. To date, no specific actions have been assigned for each member of the Rho protein family. However, differences in expression and cellular localization have been reported for these Rho proteins (38–40). The above finding also raises the possibility that Rho-effector interaction does not occur through the so-called switch regions alone because these regions are identical among three members of the Rho protein family. Indeed, Diekmann *et al.* (41) showed that a region other than switch regions of Rho was also important in elicitation of Rho-mediated stress fiber formation.

In conclusion, we have identified a new putative effector for Rho, with a region homologous to other Rho effector molecules. This region specifically binds GTP-Rho and may constitute the first consensus effector sequence for Rho small GTPases.

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